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(54) Title: SYNTHESIS OF OLIGONUCLEOTIDES

(57) Abstract

A method for the synthesis of a plurality of oligonucleotides in which an oligonucleotide is formed by sequential reactions of precursors of individual nucleotides on a support, comprising the steps of (a) forming a first oligonucleotide; (b) attaching to said first oligonucleotide a cleavable linker moiety; (c) forming a second oligonucleotide on the cleavable linker moiety; and (d) optionally cleaving the linker moiety to give the desired oligonucleotides. The invention also concerns nucleoside and non-nucleoside reagents suitable for incorporating cleavable linker moieties during automated oligonucleotide synthesis, cleavage of which produces oligonucleotides having a hydroxy or phosphate group at the 3′ and 5′ positions, and solid supports suitable for use in automated oligonucleotide synthesisers.

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SYNTHESIS OF OLIGONUCLEOTIDES

This invention relates to a method for the synthesis of oligonucleotides, to novel compounds which may be used during operation of the method, and to a solid support suitable for use in an automated oligonucleotide synthesiser.

The availability of relatively low cost synthetic oligonucleotides has been of considerable importance in the development of modern molecular biology. The polymerase chain reaction (PCR) technique (described EP 201184-A) is an example of an important, recently developed, technique which relies upon the ready availability of synthetic oligonucleotide primers. Although the basis of this technique was originally described by Kleppe et al (J. Mol. Biol. (1971), 56, 341-361), it did not assume its present importance until convenient sources of oligonucleotides became available. There is a continuing need for rapid and efficient methods for preparing and purifying oligonucleotide sequences.

Oligonucleotide sequences or derivatives thereof are routinely synthesised for use as linkers, adaptors, building blocks for synthetic genes, synthetic regulatory sequences, probes, primers and other purposes and a number of methods have been developed for producing such sequences. These methods generally rely on the initial attachment of a first suitably protected nucleoside to a solid support by a cleavable linkage followed by sequential reactions of precursors of individual nucleotides to the growing oligonucleotide strand with each addition of a precursor involving a number of chemical reactions. At present the method most generally employed for the production of a lone oligonucleotide is the method based on phosphoramidite chemistry. This is fully described by Caruthers et al in Tetrahedron Letters 1981, 22, (20) pp 1859-62 and European Patent No. 61746 and additionally by Koster et al in US Patent 4725677 (EP 152459) and by M.J. Gait ('Oligonucleotide Synthesis, a Practical Approach', IRL Press Oxford p35-81).

Several types of automated DNA synthesisers are now commercially available which enable oligonucleotides of good quality to be prepared using phosphoramidite chemistry in a reasonable amount of time - e.g. an oligonucleotide containing 30 nucleotides (a 30-mer) may be prepared routinely using a commercially available automated synthesiser in approximately 3 to 5 hours.

In response to the rapid increase in demand for oligonucleotides, improvements are desirable which will increase the throughput of such commercial synthesisers, i.e. increase the number of oligonucleotides synthesised per day.

An illustrative description of how a lone oligonucleotide may be formed by sequential reactions of precursors of the individual nucleotides on a support is provided in the protocol for the Applied Biosystems DNA Synthesiser Model 380B, particularly Section 2 thereof, which is incorporated herein by reference thereto.

We have now developed a method for the production of oligonucleotides in which more than one oligonucleotide can be synthesised on the same support in, e.g. a commercial automated synthesiser, using a cleavable linker moiety introduced as required in the growing oligonucleotide chain.

According to a first aspect of the present invention we provide a method for the synthesis of a plurality of oligonucleotides in which an oligonucleotide is formed by sequential reactions of precursors of individual nucleotides on a support, comprising the steps of (a) forming a first oligonucleotide; (b) attaching to said first oligonucleotide a cleavable linker moiety; (c) forming a second oligonucleotide on the cleavable linker moiety; and (d) cleaving the linker moiety to give the desired oligonucleotides.

As will be appreciated the first oligonucleotide is preferably formed on a support, which is preferably a solid support such as is used in automated oligonucleotide synthesis. The identity of the support is not critical and may be any of the supports used in the automated synthesis of oligonucleotides, for example, modified inorganic polymers such as those disclosed in the US Patent Specification 4,458,066, silica gels, Porasil C, kieselguhr PDMA, polystyrene, polyacrylamide, Silica

CPG (LCAA) or controlled pore glass as used in, for example, the Applied Biosystems DNA synthesiser Model 380B. The support can have a precursor of a first nucleotide cleavably attached to it, e.g. a solid support connected to an optionally protected nucleoside by means of a conventional cleavable link as described for example in the book by M.J. Gait.

The first oligonucleotide may be formed by conventional technology used for synthesising oligonucleotides, for example by using phosphoramidite chemistry on an automated oligonucleotide synthesiser as described above. The first oligonucleotide is preferably connected to the support by a hydrolysable group (e.g. a base labile group) as is known in the art.

The cleavable linker moiety may be attached to the first oligonucleotide by means of a reagent, for example a modified nucleoside, or alternatively a reagent which does not contain a nucleoside element, which is capable of connecting to said first oligonucleotide and upon which a second oligonucleotide may be formed, and which can be broken to separate the first and second oligonucleotides under conditions which do not significantly affect the oligonucleotides.

By "Conditions which do not significantly affect the oligonucleotide" it is meant conditions which do not degrade the oligonucleotide. Examples of such conditions will be apparent to those skilled in the art, for example use of neutral or alkaline pH, for example pH above 2, and conditions which are free from strong electrophiles. Strong nucleophiles, and conditions which use osmium tetroxide and other known oligonucleotide modifying agents are preferably avoided.

The first aspect of the invention may be illustrated by the formation of 2 oligonucleotides using different combinations of the phosphoramidites of 2'-deoxyadenosine (dA), 2'-deoxyguanosine (dG), 2'-deoxycytidine (dC), and 2'-deoxythymidine (dT) separated by the cleavable linker moiety L' built up sequentially in a 3' to 5' direction from the 3' hydroxy of ribose on a solid support according to the above

method described by M.J. Gait. After synthesis the sequence attached to the solid support is:

5' 3'

d(ACTTL'AGCTA) (I)

After cleavage of the linker moiety L' and the linkage by which the first oligonucleotide is attached to the solid support two oligonucleotides result:

5' 3' 5' 3'

d(ACTT) d(AGCTA)

Thus two oligonucleotides have been synthesised on a single solid support.

Accordingly, a preferred first aspect of the present invention provides a method for the synthesis of a plurality of oligonucleotides comprising the steps of:

(a) forming a first oligonucleotide on a first cleavable link attached to a solid support;

(b) attaching to the first oligonucleotide a cleavable linker moiety;

(c) forming a second oligonucleotide on the cleavable linker moiety; and

(d) cleaving the first cleavable link and the cleavable linker moiety to give a plurality of oligonucleotides.

It is preferred that the cleavable linker moiety connects the first and second oligonucleotides by a 3' and a 5' oxygen, more preferably via a phosphate, phosphite, phosphate ester, phosphite ester or H-phosphonate ester, one on each oligonucleotide.

The identity of the first cleavable link is not believed to be critical, it is preferably base labile, and may be for example any of the cleavable links used in automated oligonucleotide synthesisers, such as a link which contains a base labile ester group.

As will be apparent organic residues of the cleaved linker moiety, such as hydrocarbon chains, may remain attached to the oligonucleotides after cleavage step (d). It is, however, preferable

that after cleavage step (d) organic residues of the cleaved linker moiety do not remain attached to the oligonucleotides to avoid any adverse affects on the properties of the oligonucleotides which such residues can have.

The method of invention does not contain a step in which hybridisation of the first or second oligonucleotide with a further oligonucleotide is attempted, for example by contact with a solution containing an oligonucleotide which may be complementary to the first or second oligonucleotide because this is unnecessary.

The first aspects of the invention include repetition of steps (b) and (c) any desired number of times, for example 1 to 100 times, or preferably 1 to 5 times, to produce further oligonucleotides which are each connected through a cleavable linker moiety. As will be appreciated, when steps (b) and (c) are repeated the further oligonucleotides are formed on the cleavable linker moiety attached to the previously formed oligonucleotide and may be the same as or different to the previously formed oligonucleotides.

The cleavable linker moieties may be cleaved, e.g. by base hydrolysis, to give a mixture of individual oligonucleotides which may, if required, be purified and separated.

It will be understood that in this specification the term "oligonucleo. de" preferably includes an oligodeoxyribonucleotide, an oligoribonucleotide and analogues thereof (for example those which bear protecting groups), including those with methyl-phosphonate and phosphorothioate or phosphorodithioate diester backbones, and oligonucleotides with oligodeoxyribonucleotides, especially the 2'-oligodeoxyribonucleotides being more usually synthesised by the method of the invention. The preferred oligonucleotides are oligodeoxyribonucleotides, are essentially single stranded, and are preferably from at least two, more preferably at least 5, especially from 10 to 200 bases long.

To users of DNA synthesisers the method of the invention gives the advantage of more effective use of the apparatus and subsequently reducing the cost of production and purification of oligonucleotid s.

The DNA synthesiser can produce two or more oligonucleotides (which may be the same or different) on any one of its columns without

being re-programmed between each oligonucleotide. Thus when synthesis of one oligonucleotide is completed at a time outside the working day the synthesiser can go on to produce another without any intervention by an operative. This can significantly increase the productivity of such apparatus.

The method of the invention is particularly useful for the synthesis of primers for the Polymerase Chain Reaction (PCR) technique. At present a large proportion of oligonucleotides synthesised are for this purpose. Such primers are typically required in pairs and the method of the invention is convenient since it allows production of oligonucleotides in pairs. This is particularly an advantage when using single column synthesisers and/or for heavily used facilities for out-of hours working.

It is preferred that the precursors of the individual nucleotides are nucleoside phosphoramidites which are protected at the 5' oxygen atom and are optionally base protected. Methods of protecting nucleoside bases are known in the art, for example by a protecting group which is removable by treatment with mild acid or alkali. Adenine and cytosine may be protected by an optionally substituted N-benzoyl group and Guanine by an N-isobutyryl group. Thymine and uracil generally do not require protection. Adenocsine and Guanine may also be protected by a dimethylformamide or phenoxyacetyl group, and cytosine by an isobutyryl group. The protecting groups are desirably removed after separation of the protected oligonucleotide from the support. Cleavage of the linker moiety may be effected before, during or after the removal of the protecting groups depending upon the chemistry employed. It is preferred that the protecting groups are removable by treatment with aqueous base, particularly concentrated ammonia solution. In an embodiment of the invention the linker is cleavable under basic or alkaline conditions so that protecting group removal and cleavage of linker moieties can be effected in one step. Typical basic conditions employed, are to mix the protected oligonucleotide with concentrated ammonia, for example at around 55°C for up to 24 hours, especially from about 5 to 24 hours. It is preferred that a linker moiety is chosen such that cleavage is completed under these conditions.

Other bases, preferably volatile bases may be employed to effect cleavage. These may conveniently be organic amines in water, for example piperidine or methylamine, preferably at a concentration from 20-70%.

As examples of precursors of individual nucleotides suitable for use in the method there may be mentioned the 2-cyanoethyl-N,N-diisopropylaminophosphoramidites of 5'-dimethoxytrityl-N-4-benzoyl-2'-deoxycytidine, 5'-dimethoxytrityl-N-2-isobutyryl-2'-deoxyguanosine, 5'-dimethoxytrityl-N-6-benzoyl-2'-deoxyadenosine, and 5'dimethoxytritylthymidine.

For the synthesis of oligoribonucleotides precursors are for example the same as for oligodeoxyribonucleotides except that on the 2' position of the ribose there is a protected hydroxyl group, for example a tertiary butyl dimethyl silyloxy group or 1-[(2-chloro-4-methyl) phenyl]-4-methoxy piperidin-4-yloxy group which is abbreviated to CTMP, as described by T.S. Rao et al in Tet. Lett., 28, 4897 (1987).

In a known application of oligonucleotides, i.e. as primers for PCR, it is unimportant whether the 5' end of the oligonucleotide bears a phosphate group or hydroxy group. However, there is an increasing interest in the use of oligonucleotides having a 5' phosphate group (see e.g. Higuchi & Ockman (1989), Nucl. Acid Res. 17(14), p5865). Therefore a synthetic method that gives rise to an oligonucleotide having a 5' phosphate group is of value. A further advantageous use for oligonucleotides having a 5' phosphate group is in the chemical synthesis of genes where 5' phosphorylated oligonucleotides are desired.

Accordingly the cleavable linker moiety used in the method of the invention preferably comprise either (I) a moiety whose cleavage gives rise to OH groups at both the 5' and 3' ends of the desired oligonucleotides or (II) a moiety whose cleavage gives rise to a free 3' OH group on one oligonucleotide and a 5' phosphate group on another oligonucleotide.

Thus, in a preferred aspect the method of the present invention step (d) preferably yields desir d oligonucleotides each having at the 3' and 5' position a group selected from hydroxy and phosphate.

The reagents currently used to synthesise oligonucleotides include the protected nucleoside phosphoramidites. It would therefore be convenient if the cleavable linker moiety used in the present methods is attached by means of a modified nucleoside.

Accordingly the invention also provides a modified nucleoside reagent of general structure (II) which is capable of connecting to said first oligonucleotide and upon which a second oligonucleotide may be formed:

Z-Nuc-L'-O-PA (II)

wherein:

Nuc is a nucleoside in which the base optionally is protected;

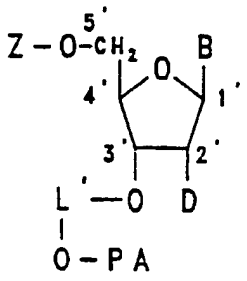
Z is a protecting group attached to the 5' oxygen of Nuc;

-O-PA is a phosphoramidite group, a phosphate ester group, a H-phosphonate group or other group capable of conversion to a phosphodiester group; and

L' is a cleavable linker moiety, which is preferably a hydrolysable separating group attached to the 3' oxygen of Nuc.

By the term "hydrolysable" it is meant that L' may be cleaved or split into two or more parts by treatment with base, for example with aqueous alkali, ammonium hydroxide or piperidine.

The modified nucleoside of formula Z-Nuc-L'-O-PA is preferably of Formula (III):



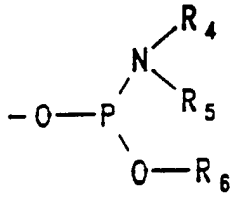
(III)

wherein Z, L' and -O-PA are as hereinbefore described, B is an optionally protected base such as optionally protected uracil, thymine, cytosine, adenine or guanine, or analogues thereof, and D is H or a protected hydroxyl group.

As examples of phosphate ester groups and H-phosphonate groups these may be mentioned groups which, in the free acid form, are respectively of formula:

wherein Z_3 is a protecting group, preferably a base labile protecting group, for example 2-chlorophenyl or 2,4-dichlorophenyl.

Preferably, -O-PA is a phosphoramidite of general structure:



wherein R_4 and R_5 are each independently optionally substituted alkyl, especially C_{1-4}-alkyl; optionally substituted aralkyl, especially optionally substituted benzyl; cycloalkyl and cycloalkylalkyl containing up to ten carbon atoms, such as cyclopentyl or cyclohexyl; or R_4 and R_5 taken together with the nitrogen atom to which they are attached form an optionally substituted pyrollidine or piperidine ring or R_4 and R_5 when taken together with the nitrogen atom to which they are attached form a saturated nitrogen heterocycle which optionally includes one or more additional hetero atom from the group consisting of nitrogen, oxygen and sulphur. R_4 and R_5 are preferably iso-propyl.

R_6 represents a hydrogen atom or a protecting group, for example a phosphate protecting group. As examples of phosphate protecting groups there may be mentioned optionally substituted alkyl groups, for example methyl, 2-cyanoethyl, 2-chlorophenyl, 2,2,2-trihalo-1,1-dimethyl ethyl, 5-chloroquin-8-yl, 2-methylthioethyl and 2-phenylthioethyl groups in which the phenyl ring is optionally substituted, for example by a group selected from halogen, eg. chlorine, or NO_2. Preferably R_6 is methyl or, more preferably, 2-cyanoethyl.

Nuc in the structure II above represents the conventional nucleoside and deoxynucleosides (deoxy)cytidine, (deoxy)adenosine, (deoxy)guanosine, (ribo)thymidine or (deoxy)uridine as well as analogues thereof. The base portion of the nucleoside optionally is protected by a protecting group. Thus, for example, the amine substituent in adenine, cytosine and guanine may be protected by any of the protecting groups used in the art (for example, as described in E Ohtsuka et al, Nucleic Acids Research, (1982), 10, 6553-6570).

Moreover appropriate base protecting groups are apparent to nucleotide chemists and include particularly isobutyryl and optionally substituted benzoyl; the isobutyryl group being particularly appropriate as a protecting group for guanine and the optionally substituted benzoyl group being particularly appropriate as a protecting group for cytosine and adenine. Nucleosides in which the base is protected include, for example, N^4-benzoylcytosine, N^6-benzoyladenine and N^2-isobutyrylguanine. It will be appreciated that not all bases will require protection, for example thymine and uracil.

Z in the above formulae represents a protecting group for the 5'-hydroxyl group of the nucleoside, especially an acid labile protecting group. Suitable protecting groups will be apparent to those skilled in the art and include those discussed in 'Protective Groups in Organic Synthesis' by T.W. Greene, Wiley Interscience. Examples of such protecting groups include, tetrahydropyranyl e.g. tetrahydropyran-2-yl, 4-methoxytetrahydropyranyl e.g. 4-methoxytetrahydropyran-2-yl, methoxytrityl (preferably for oligoribonucleotide

synthesis only), dimethoxytrityl, pixyl, isobutyloxycarbonyl, t-butyl dimethylsilyl and like protecting groups. Preferably, Z is dimethoxytrityl.

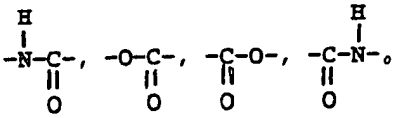
As will be understood when -O-PA is a H-phosphonate or a phosphoramidite these are oxidised to respectively a phosphate diester or phosphate tri-ester groups during operation of the method, for example using aqueous iodine or peroxide. In the case of H-phosphonate the oxidation is preferably performed after step (c) and before step (d), whilst in the case of phosphoramidite it is preferably performed during step (a) and step (c).

In a modified nucleoside reagent of Formula (II) L' preferably comprises or consists of three sections (i), (ii) and (iii) which have the structures discussed below.

Section (i) suitably comprises a group which upon cleavage, for example by hydrolysis, leads to the generation of a 3' hydroxyl group at the 3' terminus of the oligonucleotide to which it was previously attached. As examples of such groups there may be mentioned carbonyl, CONH and imidate groups. Preferably section (i) is a carbonyl, CONH or $-C(=NH_2^+)-$ group which combines with the oxygen atom of a 3' hydroxyl group of an oligonucleotide to give an hydrolysable moiety, especially a carboxylic acid ester moiety.

Preferably therefore section (i) is a  group.

Section (ii) can be any spacer group, preferably a spacer group compatible with automated oligonucleotide synthesis, for example a divalent organic spacer group or a hydrocarbon spacer group. Conveniently section (ii) is a divalent organic spacer group, for example of 2 to 15 atoms in length, preferably 2 to 6 atoms in length. The preferred divalent organic spacer group comprises or consists of one or more substituted or unsubstituted methylene groups optionally interrupted by other groups such as 1,2-,1,3-, or 1,4-phenylene, cyclohex-1,4-ylene, -S-, -O-, $-SO_2-$, -NHCONH, -S-S-,



The optional interruptions are introduced in order to facilitate synthesis of the reagent and/or to provide elements to reduce the possibility of the spacer group folding in on itself. A particularly convenient method of extending from the 3' hydroxyl of an oligonucleotide is by reaction with succinic anhydride and accordingly a separating group in which section (i) is -CO.- and section (ii) is or contains the group $-(CH_2)_2CO.-O-$ is especially suitable.

Section (iii) may comprise a group capable of giving rise to beta-elimination of a phosphate ester group. This group can be of two types, Type A or Type B:

Type A is of the structure:

$$-Q2-CHR_1-CR_2R_3-$$

where Q2 is a electron withdrawing group such as $-SO_2-$ and R_1, R_2 and R_3 are each independently H or a non-electron withdrawing group such as alkyl, especially C_{1-4}-alkyl, or a substituent that is not itself a leaving group in a beta-elimination reaction and does not otherwise interfere when a phosphate ester group, introduced by means of -O-PA, is eliminated. Alternatively, R_1 may be an electron-withdrawing group.

Type B is of the general formula:

$$\begin{array}{l} Q1 \\ | \\ -CH-CR_2R_3- \end{array}$$

where Q1 is an electron withdrawing group, for example -F, $-CF_3$, $-NO_2$, -phenyl, aryl (for example phenyl, substituted phenyl, or preferably p-nitrophenyl), cyano, $-SO_3R$ (R=alkyl) and R_2 and R_3 are as defined above.

It is preferred that R_1, R_2 and R_3 are each independently H or C_{1-4}-alkyl, more preferably H or methyl, especially H.

Alternatively section (iii) is a group of formula $R_9-CR_7(OZ^2)-CR_7R_8-$ or $-CO.-O-CR_7R_{11}-CR_7R_{10}-$ wherein;

each R_7 independently is H or C_{1-4}-alkyl;

one of R_8 & R_9 is a single bond and the other is H or C_{1-4}-alkyl;

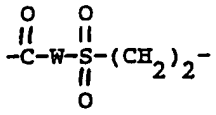
R_{10} & R_{11} are each independently H or C_{1-4}-alkyl or R_{10} together with R_{11} and the carbon atoms to which they are attached form an optionally substituted 4,5,6 or 7 membered alicyclic or heterocyclic ring; and

Z^2 is a protecting group, preferably a base labile protecting group.

It will be appreciated that the group (i) may be sufficiently electronegative to serve as element Q2 in group (iii). In such instances, the 3' oxygen of Nuc is attached directly to group (iii) and group (ii) is obviated.

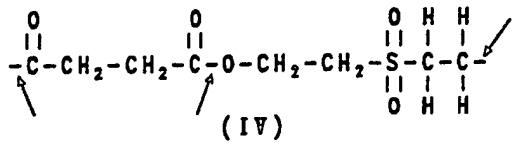
The sections are preferably linked together in the order (i), (ii), (iii) with (i) and (iii) being connected to nucleoside (Nuc) and -O-PA respectively, as shown in structure (III).

Preferably the cleavable linker moiety L' is of the formula:



wherein W is a divalent organic spacer group as defined in Section (ii), especially $-CH_2CH_2-CO.OCH_2CH_2-$.

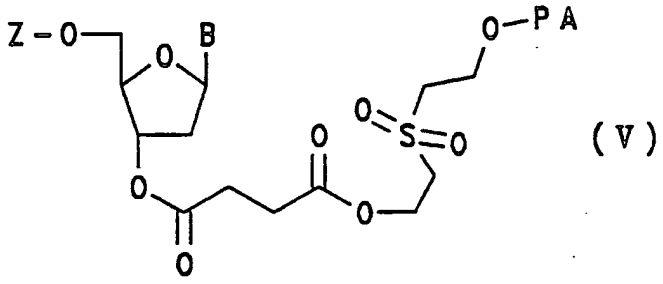
It is preferred that L' is of formula (IV):



(IV)

When the method according to the first aspect of the invention has been performed to give a plurality of oligonucleotides connected by a group or groups of formula (IV) treatment with base cleaves at the points marked with arrows on (IV) to give a plurality of oligonucleotides, one of which is 5' phosphorylated.

The modified nucleoside is preferably of the formula (V):



(V)

wherein Z, B and PA are as hereinbefore defined.

Compounds of Formula (II) or (III) wherein -O-PA is a phosphoramidite group may be prepared by reacting a compound of formula Z-Nuc-L'-OH with a compound of formula X^1-PA in CH_2Cl_2 using diisopropylethylamine as base, wherein PA is a phosphoramidite as defined above for -O-PA except that -O- is absent, and X^1 is a leaving group, for example Cl or Br.

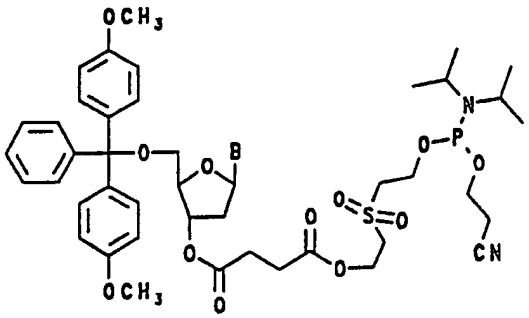
When -O-PA in Formula (II) or (III) is a phosphate ester group, the compound of Formula (II) or (III) may be prepared by reaction of a compound of formula Z-Nuc-L'-OH with the triazolide of the corresponding free phosphate ester using a method analogous to that described in the above book by M.J. Gait.

When -O-PA in Formula (II) or (III) is a H-phosphonate group the compound of Formula (II) or (III) may be prepared by reaction of a compound of formula Z-Nuc-L'-OH with PCl_3 in the presence of 1,2,4-triazole using a method analogous to that described by B.C. Froeher et al, in Nucleic Acids Research, (1986), 14, 5399-5407.

The compound of the formula Z-Nuc-L'-OH may be prepared in two steps by reaction of a compound of formula Z-Nuc-OH with the anhydride of a suitable bifunctional carboxylic acid, for example succinic acid, followed by coupling of the acid derivative so produced with a suitable dihydroxy compound, for example 2,2'-sulfonyl diethanol. For this coupling of the carboxylic acid derivative with the dihydroxy compound, the carboxylic acid may be activated toward reaction with a hydroxyl group by methods known in the art, for example by in situ formation of the symmetrical anhydride by the condensation of two molecules of the carboxylic acid derivative via the intercession of a coupling agent such as for example 1,3-dicylclohexyl carbodiimide. The reaction of the hydroxy compound with the activated carboxylic acid derivative may be performed in an aprotic solvent in the presence of one molar equivalent of base. The compound of formula Z-Nuc-L'-OH so produced is preferably purified from the reaction mixture by some suitable means, for example chromatography.

The compound of formula Z-Nuc-OH may be prepared by reaction of a compound of formula HO-Nuc-OH with a compound of formula $Z-X^1$ (wherein X^1 and Z are as defined above) preferably in an aprotic solvent in the presence of a molar equivalent of base. Preferably $Z-X^1$ is a compound that reacts preferentially at only one of the two (or three if HO-Nuc-OH is a ribonucleoside) available hydroxyl groups. Preferably, $Z-X^1$ reacts selectively with the primary hydroxyl at the 5'- position of HO-Nuc-OH.

A convenient modified nucleoside is of formula (VI):



(VI)

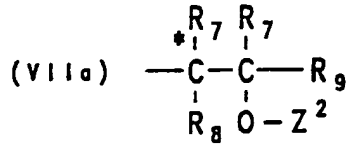
wherein B is as hereinbefore defined.

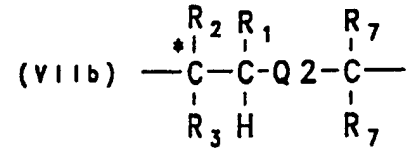
It will be appreciated that in the example of structure I, the cleavable linker moiety L' can be either introduced by means of a reagent (for example of structure II) which comprises a nucleoside that will become the 3' nucleotide of the second oligonucleotide or by means of a reagent that does not contain a nucleoside element. The modified nucleosides of general structure (II) are of great value for introducing a cleavable linker moiety as described in the method of the invention. However, five such nucleosides are required depending on whether the 3' nucleoside of a second desired nucleotide is A, G, T, C or U. For economy and convenience it would be desirable to have a single reagent, which does not contain a nucleoside element, which is capable of connecting the first and second oligonucleotides together and is compatible with phosphoramidite chemistry or other chemistry used in oligonucleotide synthesis, for example in DNA synthesisers, and is capable of being completely removed from the oligonucleotides, for example by treatment with ammonium hydroxide.

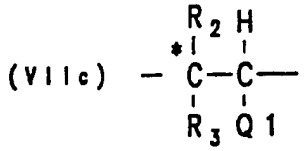
Accordingly the present invention provides a compound of Formula (VII):

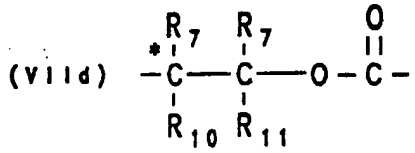
$$Z^1\text{-}O\text{-}A^1\text{-}E\text{-}A^2\text{-}O\text{-}PA \qquad \text{(VII)}$$

wherein A^1 and A^2 are each independently of the formula (VIIa), (VIIb), (VIIc) or (VIId) wherein the carbon atom marked with an asterisk is attached to the oxygen atom shown in formula (VII);

(VIIa) 

(VIIb) 

(VIIc) 

(VIId) 

Z^1 is a protecting group;

R_1, R_2, R_3, Q1, Q2 and -O-PA are as hereinbefore defined;

each R_7 independently is H or C_{1-4}-alkyl;

one of R_8 and R_9 is a single bond by means of which the group of formula (VIIa) is attached to E, and the other is H or C_{1-4}-alkyl;

Z^2 is a protecting group, preferably a base labile protecting group;

R_{10} & R_{11} are each independently H or C_{1-4}-alkyl or R_{10} together with R_{11} and the carbon atoms to which they are attached form an optionally substituted 4, 5, 6 or 7 membered alicyclic or heterocyclic ring;

E is a single covalent bond or a spacer group; and provided that when A^1 and A^2 are both of Formula (VIId) E is a spacer group.

The protecting group represented by Z^1 is preferably an acid labile protecting group, more preferably an acid labile protecting group listed above for Z, especially dimethoxytrityl.

When Z^2 is a base labile protecting group it is preferably selected from the base labile protecting groups disclosed in the abovementioned book by T.W. Green, especially a silyl group, for example t-butyl dimethylsilyl, or more preferably an acyl group such as a C_{1-4}-alkanoyl group or especially an optionally substituted benzoyl group which has been found, surprisingly, to result in particularly stable compounds of Formula (VII).

When any of R_1, R_2, R_3, R_7, R_8, R_9, R_{10} and R_{11} is H or C_{1-4}-alkyl it is preferably methyl, more preferably H. Q_2 is preferably $-SO_2-$.

When E is a spacer group it is preferably a spacer group as hereinbefore defined in section (ii), more preferably an optionally substituted alkyl, alicycic or aryl group, especially phenylene or an alkyl group containing up to 6 carbon atoms.

The preferred alicyclic ring is a 5 or 6 membered ring, for example a cyclohexyl or cyclopentyl ring. The preferred heterocyclic ring is a 5 or 6 membered ring, for example furanyl or pyranyl ring.

When A^1 and A^2 are both of Formula (VIIa) it is preferred that E is a single covalent bond, $-(CH_2)_m-$, $-CO.NH(CH_2)_mNH-CO.-$, $-O-CO.-G-CO.-O-$ or $-CO.-O-G-O-CO.-$ wherein G is $-(CH_2)_m-$, aryl, especially phenyl, or an alicyclic group such as cyclohexyl, cyclohexylmethylene or cyclopentyl, and each m independently has a value of from 1 to 6, preferably 2 to 6, especially 2.

When A^1 and A^2 are both selected from Formula (VIIb) or (VIIc) it is preferred that E is of formula $-(CH_2)_m-$ or $-(CH_2)_m-O-CO.-G-CO.-O-(CH_2)_m-$ wherein m and G are as hereinbefore defined.

When A^1 and A^2 are both of Formula (VIId) it is preferred that E is of formula G as hereinbefore defined, especially $-(CH_2)_2-$ or phenyl.

When A^1 is of Formula (VIIa) and A^2 is of Formula (VIIb) or (VIIc) it is preferred that E is of formula $-(CH_2)_m-$, $-G-CO.-O-(CH_2)_m-$ or $-G-O-CO.-(CH_2)_m-$ wherein m and G are as hereinbefore defined.

When A^1 is of Formula (VIIa) and A^2 is of Formula (VIId) it is preferred that E is of formula $-(CH_2)_m-$, $-O.CO.-G-$ or $-G-CO.-O-G-$ wherein m and G are as hereinbefore defined.

When A^1 is of Formula (VIIb) or (VIIc) and A^2 is of Formula (VIIa) it is preferred that E is of formula $-(CH_2)_m-$, $-(CH_2)_m-O-CO.-G-$ or $-(CH_2)_m-CO.-O-G-$ wherein m and G are as hereinbefore defined.

When A^1 is of Formula (VIIb) or (VIIc) and A^2 is of Formula (VIId) it is preferred that E is of formula $-(CH_2)_m-$ or $-(CH_2)_m-OCO.-G-$ wherein m and G are as hereinbefore defined.

When A^1 is of Formula (VIId) and A^2 is of Formula (VIIa) it is preferred that E is of formula $-(CH_2)_m-$, $-G-O-CO.-G-$ or $-G-CO.-O-G-$ wherein m and G are as hereinbefore defined.

When A^1 is of Formula (VIId) and A^2 is of Formula (VIIb) or (VIIc) it is preferred that E is of formula $-(CH_2)_m-$ or $-(CH_2)_m-OCO.-G-$ or $-(CH_2)_m-CO.-O-(CH_2)_m-$ wherein m and G are as hereinbefore defined.

It is preferred that A^1 and A^2 are each independently selected from Formula (VIIa), (VIIb) and (VIId) wherein the carbon atom marked with an asterisk is attached to the oxygen atom shown in Formula (VII).

As examples of groups represented by Formula (VIIa) there may be mentioned $-{}^*CH_2-CH(OZ^2)-$, $-{}^*CH_2-\underset{|}{C}(OZ^2)-CH_3$, and $-\underset{|}{CH}-CH(OZ^2)-CH_3$.

As examples of groups represented by Formula (VIIb) there may be mentioned $-*CH_2CH_2-SO_2-CH_2-$ and $-*CHCH_3-CH_2-SO_2-CH_2-$.

As examples of groups represented by Formula (VIIc) there may be mentioned $-*CH_2CHF-$ and $-*CH_2CH.CF_3-$.

As examples of groups represented by Formula (VIId) there may be mentioned $-*CH_2CH_2-OCO.-$ and $-*CH(CH_3)CH_2-OCO.-$.

The compounds of formula (VII) are suitable reagents for attaching a cleavable linker moiety of formula $-A^1-E-A^2-$ between a first and second oligonucleotide as described by the method of the invention. Under suitable conditions, for example treatment with ammonium hydroxide, the compounds cleave to give the desired oligonucleotides free from any organic residue of the compound of formula (VII). This is of particular value where oligonucleotides are desired with free or phosphated 3' or 5' termini.

The utility of compounds of Formula (VII) can be illustrated by reference to the preparation of the sequence of Formula (I) as discussed above. For example, when L^1 in Formula (I) is derived from a compound of Formula (VII) in which A^2 is of Formula (VIIa) or (VIId) the oligonucleotide of formula d(AGCTA) results having a 5'-OH group, and when A^2 is of Formula (VIIb) or (VIIc) d(AGCTA) results having a 5'-phosphate group. Accordingly, by appropriate selection of A^2 in a compound of Formula (VII) from (VIIa), (VIIb), (VIIc) and (VIId) the method of the invention provides the great benefit of enabling one to select whether the first, second, and subsequent oligonucleotides prepared according to the method of the invention have a hydroxy group at the 3' position and a hydroxy or phosphate group at the 5' position.

Compounds of Formula (VII) wherein -O-PA is a phosphoramidite may be prepared by reacting a compound of formula $Z^1-O-A^1-E-A^2-OH$ with a compound of formula X^1-PA in CH_2Cl_2 using di(N-isopropyl)ethylamine as base. PA is preferably a phosphoramidite as defined above for -O-PA except that -O- is absent, and Z^1, A^1, E and A^2 are as hereinbefore defined, and X^1 is a leaving group, for example Cl or Br.

When -O-PA in Formula (VII) is a phosphate ester group as hereinbefore defined the compound of Formula (VII) may be prepared by reaction of a compound of formula Z^1-O-A^1-E-A^2-OH with the triazolide of the corresponding free phosphate ester using a method analogous to that described in the above book by M.J. Gait.

When -O-PA in Formula (VII) is a H-phosphonate group as hereinbefore defined the compound of Formula (VII) may be prepared by reaction in a compound of formula Z^1-O-A^1-E-A^2-OH with PCl_3 in the presence of 1,2,4-triazole using a method analogous to that described by B.C.Froehler et al, Nucleic Acid Research, (1986), 14, 5399-5407.

The compound of formula Z^1-O-A^1-E-A^2-OH may be prepared by deprotection of a compound of formula Z^1-O-A^1-E-A^2-O-TBDMS, wherein TBDMS is a t-butyldimethyl silyl group (which is removable using tetrabutyl ammonium fluoride in THF) or other protecting group which is removable under neutral conditions.

When A^1 or A^2 is of formula (VIIa) the compound of formula Z^1-O-A^1-E-A^2-O-TBDMS may be prepared by reaction of the compound of formula Z^1-O-A^3-E-A^4-O-TBDMS (wherein A^3 is as defined for A^1 except that Z^2, when present, is H and A^4 is as defined for A^2 except that Z^2, when present, is H) with a compound of formula Z^2-X^1 wherein X^1 is a leaving group, for example Cl or Br (e.g. a C_{1-4}-alkanoyl halide such as acetyl chloride or propanoyl bromide, or an optionally substituted benzoylhalide). The compound of formula Z^1-O-A^3-E-A^4-O-TBDMS may be prepared by reaction of Z^1-O-A^3-E-A^4-OH with TBDMS-Cl. Z^1-O-A^3-E-A^4-OH may be prepared by reaction of HO-A^3-E-A^4-OH with Z^1-Cl, preferably in an aprotic solvent and with 1 equivalent of base such as pyridine, and removing any Z^1-O-A^3-E-A^4-O-Z^1 by a standard purification technique such as chromatography.

When A^1 and A^2 are each of formula (VIIb), (VIIc) or (VIId) the compound of formula Z^1-O-A^1-E-A^2-OH may be prepared by reaction of a compound of formula Z^1-O-A^1-E-CO_2H with a compound of formula HO-A^2-OH, preferably in an aprotic solvent using a suitable condensing agent such as th aforementioned DCCI or 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide.

The compound of formula Z^1-O-A^1-E-CO_2H may be prepared by the reaction of the compound of formula Z^1-O-A^1-OH with an activated form of the compound of formula HO_2C-E-CO_2H, preferably an aprotic solvent in the presence of a molar equivalent of base. The dicarboxlic acid may be activated to attack by the hydroxyl group by being present as the acid anhydride, the acid chloride or some other suitable derivative, or the reaction may be mediated by the presence of a coupling agent as described above.

The compound of formula Z^1-O-A^1-OH may be prepared by the reaction of the compound of formula HO-A^1-OH with Z^1-Cl (or some other suitably activated form of Z^1) in an anhydrous aprotic solvent in the presence of a molar equivalent of base.

In the above processes for the preparation of the compound of Formula (VII), and precursors thereof, Z^1, A^1, E. A^2, O, PA and Z^2 are as hereinbefore defined except where stated otherwise and DCCI is 1,3-dicylohexylcarbodiimide.

According to a further aspect of the invention there is provided a compound comprising two or more oligonucleotides linked, preferably by 3' and 5' oxygen atoms, by a group or groups containing a cleavable linker moiety of formula -A^1-E-A^2- or -L'- wherein A^1, E, and L' and A^2 are as hereinbefore defined. It is preferred that the cleavable linker moiety and formula -A^1-E-A^2- or -L'- is connected to each oligonucleotide via a H-phosphonate, phosphate, phosphite, phosphate ester or phosphite ester linkage. It is preferred that one of the oligonucleotides is connected to a support.

H-phosphonate linkages are of formula -HP(=O)-, preferred phosphate ester linkages are of formula -P(=O)-OR_6.- and preferred phosphite linkages are of formula -P(-OR_6)- wherein R_6 is as hereinbefore defined.

One feature of most of the currently used methods for synthesising a lone oligonucleotide on a support is that the synthesis starts with a commercially available support containing the first (the

3') nucleoside already attached. This is primarily because of the need to provide a cleavable link between the lone oligonucleotide and the solid support. Hitherto this has prevented the use of protected nucleoside precursors as the sole means for introducing nucleotide elements into the oligonucleotide. There is a need for a method whereby oligonucleotides can be synthesised on a support without the use of a support with the first nucleoside already attached.

We have found that a compound of Formula (II) or (VII) may also be used to convert a support which does not have a first cleavable link attached to it to a support which does have a first cleavable link attached.

Accordingly, a further aspect of the present invention comprises a method for the preparation of solid support bearing a cleavable link by condensation of a solid support which does not bear a cleavable with a compound of Formula (II) or (VII) as hereinbefore defined.

In this further aspect the solid support is preferably one of the conventional supports which has hydroxyl or amino groups, preferably hydroxyl groups, for example one of the aforementioned solid supports used in automated oligonucleotide synthesis. Reaction with a reagent of Formula (II) or (VII) may be performed by analogous method to these which are known. Depending on the nature of -O-PA in the reagent (II) or (VII), the cleavable link may be introduced by means of an automated nucleic acid synthesiser, in the same manner as for the nucleotide precursors. In this aspect of the invention use of a reagent of Formula (II) or (VII) which does not contain a beta-elimination moiety (eg, where A^2 is of Formula (VIIa) or (VIId) are preferred, since these do not give rise after cleavage to undesirable phosphorylation of the solid support. A convenient feature of the reagents which do not contain the beta-elimination group is that the hydroxyl group of hydroxyl containing supports is re-generated allowing the possibility of re-use of the support for the synthesis of further oligonucleotides.

A general advantage of this further aspect of the invention is the avoidance of purchasing or synthesising the support with the first nucleoside attached. This is especially advantageous for the synthesis of oligonucleotides of non-conventional structure where the support containing the first attached nucleoside may not be readily obtainable.

A still further aspect of the present invention provides a solid support, suitable for use in an automated oligonucleotide synthesiser, of Formula (VIII) or (IX):

$$SUP\text{-}P^1\text{-}L'\text{-}Nuc\text{-}Z \qquad (VIII)$$

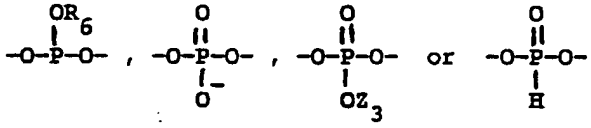
$$SUP\text{-}P^1\text{-}A^2\text{-}E\text{-}A^1\text{-}O\text{-}Z^1 \qquad (IX)$$

wherein;

L', Nuc, Z, A^2, E, A^1 and Z^1 are as hereinbefore defined;

SUP is a solid support, preferably a solid support having hydroxy or amino groups suitable for use in an automated oligonucleotide synthesiser; and

P^1 is of the formula:



wherein;

R_6 and Z_3 are as hereinbefore defined.

SUP is preferably one of the aforementioned solid supports used in automated oligonucleotide synthesis.

The invention is illustrated by the following non-limiting examples:

Example 1

Preparation of reagent M^1

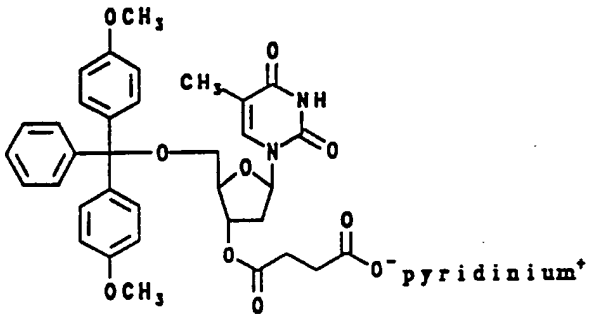
5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidin-3'-yl 2-(2-[(2-cyanoethoxy)N,N-(diisoproplyamino)phosphanyloxy]ethyl-sulfony-1)ethyl succinate.

This was synthesised using the steps numbered 1 to 3 described below.

Reagent M^1 is of formula (VI) wherein B is thymidinyl.

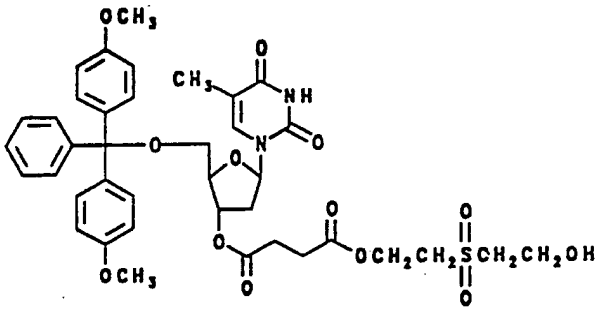
Step 1:

Pyridinium5'-O(4,4'-dimethoxytrityl)-2'-deoxythymidin-3'-yl 2-pyridinium succinate.



This compound was prepared by the method described by Gait et al in Nucleic Acids Research (1980), 8(5), 1090.

Step 2:



5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidin-3'-yl 2-(2-hydroxyethyl-sulfonyl)ethyl succinate.

5'-O(4,4'-dimethoxytrityl)-2'-deoxythymidin-3'-yl 2-pyridinium succinate (3.0 g, 4.2 mmoles) was added to a solution of dicyclohexyl-carbodiimide (0.43 g, 2.1 mmoles) in dichloromethane (40 ml) under an atmosphere of argon. The reaction mixture was stirred at room temperature for 40 minutes and, after filtration, reduced to dryness under vacuum. The residue was dissolved in dry, distilled

pyridine (30 ml) and sulphonyldiethanol (0.38 g, 3.14 mmole dried by azeotropic distillation with toluene below 45°C (caution explosion hazard) was added to the solution under an atmosphere of argon. The reaction mixture was stirred at room temperature overnight and then the solvent was removed under reduced pressure. The crude product (2.7 g) was obtained as a pale yellow foam after the residue was azeotroped with dry toluene (2 x 30 ml). The product, (0.38 g, 0.5 mmole, 12.5%) was obtained as a white solid after chromatography on silica gel (Merck Art No. 9385, 250 g) with eluant of methanol:dichloromethane (3:47, 2000 ml).

^{1}H nmr (delta) (CDCL3,400 MHz); 8.56 (1H,s,NH), 7.25 (9H,m, aromatic protons), 6.83 (4H,d, aromatic protons), 6.38 (1H,dd,H-1'), 5,46 (1H,m,H-3'), 4.58 (2H,t,2H-9), 4.15 (1H,m,H-4'), 4.86 (2H,t,2H-12), 3.80 (6H,s,2 x OCH3), 3.47 (4H,m,2H-10 and 2H-5'), 3.27 (2H,t,2H-11), 2.90 (1H,t,OH), 2.68 (4H,s,2H-7 and 2H-8) 2.47 (2H,m,2H-2'), 1.38 (3H,s,CH3).

Step 3:

5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidin-3'-yl 2-(2-[(2-cyanoethoxy)N,N-(diisopropylamino)phosphanyloxy]ethyl sulfonyl)ethyl succinate (ie, Reagent M^1).

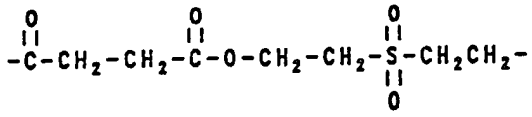
5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidin-3-yl 2-(2-hydroxyethylsulfonyl)ethyl succinate (200 mgs, 0.26 mmoles) was added to a solution of N,N-diisopropylethylamine (1.0 mmoles, 0.13 g, 0.17 ml) in dry dichloromethane (5 mls). The stirred solution was maintained under an atmosphere of argon at room temperature and a solution of chloro-N,N-diisopropylamino-O-cyanoethylphosphine (0.26 mmoles, 61.5 mgs, 41 microlitres) in dichloromethane (1 ml) was added over a period of 10 minutes. After 60 minutes a further addition of chloro-N,N-diisopropylamino-O-cyanoethylphosphine (0.13 mmoles, 30.75 mgs, 20.5 microlitres) was made. The crude product was obtained by evaporation of the solvent under reduced pressure and the product was isolated as a colourless oil (45.3 mgs, 18%) from chromatography on silica gel (Merck Art No 9385, 13g) with eluant of triethylamine: ethylacetate:dichloromethane (4:3:3, 100 mls).

^{1}H nmr (delta) (CDCL3, 400 MHz); 7.61 (1H,s,NH), 7.25 19H,m, aromatic protons), 6.83 (4H,d, aromatic protons), 6.4 (1H,dd,H-1'), 5.48 (1H,m,H-3'), 4.58 (2H,t,H-9), 4.17 1H,m,H-4'), 4.08 (2H,m,2H-12), 3.82 (2H, complex m, OCH2CH2CN), 3.6 (2H,m,2CH(CH3)2) 3.48 (4H,m,2H-5' and 2H-10), 3.30 (2H, complex m, 2H-11), 2.68 (6H,m,2H-7, 2H-8, CH2CN) 2.45 (2H,m,2H-2'), 1.36 (3H,s,CH3), 1.18 (12H,dd,2CH (CH3)2).

Example 2

The product of Preparation 3 above was used as described below to introduce the cleavable linker moiety L'in the synthesis of two oligonucleotides from one nucleoside bound to a solid support

The fully protected oligodeoxyribonucleotides of sequence TCTAACAGCTGATCTL'CAGCTGATCC was prepared on an Applied Biosystems 380B DNA Synthesiser from 5'-dimethoxytrityl-N-4-benzoyl-2'-deoxycytidine bound to controlled pore glass via 3' -OH and a succinylglycylglycylamino-propyl spacer (Applied Biosystems Inc) and the 2-cyanoethyl-N,N-diisopropylaminophosphoramidites of 5'-dimethoxytrityl-N-4-benzoyl-2'-deoxycytidine, 5'-dimethoxytrityl-N-2-isobutyryl-2'-deoxyguanosine, 5'-dimethoxytrityl-N-6-benzoyl-2'-deoxyadenosine, 5'dimethoxytritylthymidine (Cruachem Ltd) and 5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidin-3'-yl 2-(2-[(2-cyanoethoxy)N,N-(diisopropylamino)phosphanyloxy]ethylsulfonyl) ethyl succinate (Reagent M^1). In this example L' is represented by the structure:



As will be understood, the 3' oxygen at the end of one oligonucleotide is attached directly to the left hand side of L' (as drawn) and the 5' oxygen at the end of the other oligonucleotide is attached to the right side of L' via the phosphite linkage $-O-P(=O)(-OCH_2CH_2CN)-$.

5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidin-3'-yl 2-(2-[(2-cyanoethoxy)N,N-(diisoproplyamino)phosphanyloxy]ethyl-sulfonyl)ethyl succinate (90 mgs) in 1,2-dichloroethane:anhydrous acetonitrile (10:9, 0.95 ml 0.1 M) was used in place of a normal phosphoramidite at position 5 on our Applied Biosystems 380B DNA Synthesiser to introduce reagent M^1 which will introduce TL' in the sequence above. The procedure consisted briefly of: (1) removal of the dimethoxytrityl group with 3% trichloroacetic acid in dichloromethane; (2) coupling of 5'-O-(4,4'-dimethoxytrityl)-2'- deoxythymidin-3'-yl 2-(2-[(2-cyanoethoxy)N,N-(diisopropylamino) phosphanyloxy]ethylsulfonyl) ethyl succinate (0.1 M solution in 1,2 dichloroethane:anhydrous acetonitrile, 10:9) activated by tetrazole for 1 minute; (3) iodine oxidation of the intermediate phosphite linkage to a phosphate linkage; (4) a capping step with acetic anhydride.

The detritylated oligodeoxyribonucleotide sequence was cleaved from the solid support and also cleaved at the cleavable link moiety (L') and completely deprotected by treatment with ammonium hydroxide solution (sp.gr. 0.88) for 16h. at 55°C. The ammonium hydroxide solution was evaporated and the residue was dissolved in sterile water (1 ml). This product was analysed by hplc using a Partisil SAX 10 micron column (Jones Chromatography) with eluant A, 60% formamide and eluant B 0.3 M potassium dihydrogen orthophosphate in 60% formamide with a gradient of 0-85% eluant B in 30 minutes. The chromatography revealed the presence of three oligonucleotide sequences in approximately equal amounts which eluted from the column after 10.5, 13.9 and 14.8 minutes. These products were identified by comparing their elution times with those of independently synthesised oligonucleotide sequences and were shown to be oligonucleotides of

sequence: dCAGCTGATCC (elution time 10.5 minutes); - 5'-phosphorylated dCAGCTGATCC (elution t: e 13.9 minutes) and dTCTAACAGCTGATCT (elution time 14.8 minutes). The sequence eluting after 10.5 minutes was a result of a partial coupling reaction of reagent M^1 to the oligonucleotide sequence CAGCTGATCC and termination of further chain extension by the capping procedure.

Example 3

The method of the invention was used in the synthesis of two oligodeoxyribonucleotide P.C.R. primers from one deoxynucleoside bound to a solid phase.

The fully protected oligodeoxyribonucleotide of sequence

5' 3' 5' 3'

dCTATTCAAAATCGGAGCTCTAAGATL'TAGGGATTTGATTTTACGAGAGAGA

was prepared on an Applied Biosystems 380A DNA Synthesiser from 5'-(4,4'-dimethoxytrityl)-N-6-benzoyl-2'-deoxyadenosine bound to a controlled pore glass support via 3'-OH and a succinylglycylglycyl-aminopropyl spacer. The two P.C.R. primers were obtained from identical reagents, synthesis procedures, cleavage and deprotection procedures to those described in Example 2, except that reagent M was introduced by treatment with two, 2.5 minute activations with tetrazole.

After completion of the synthesis, cleavage from the solid support, cleavage of the cleavable linker moiety and removal of the base protecting groups were all achieved by incubation in ammonia solution, according to normal oligo synthesis protocols. The ammoniacal solution containing the pair of oligomers was then lyophilized, the residue was redissolved in 1 ml of water and the DNA concentration was determined spectrophotometrically. The mixture of oligodeoxynucleotides thus produced is referred to hereinafter as "primer mix 1".

Two other PCR primers were prepared individually by standard means to serve as a comparison for the efficiencies of the PCR's performed using primer mix 1. The sequences of the primers are:

Oligo 1: 5'-CTATTCAAAATCGGAGCTCTAAGAT 3'

Oligo 2: 5'-TAGGGATTTGATTTTACGAGAGAGA 3'

Polymerase Chain Reaction assays were set up as follows: In a total volume of 100 microlitres, each tube contained final concentrations of 50 mM KCl, 10 mM Tris.Cl (pH 9.0), 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton X-100. Each tube also contained a final concentration of 50 micromolar each of dATP, dGTP, dCTP, dTTP. In each tube was contained 30 ng of Chlamydia trachomatis (Serovar L2) genomic DNA and 2.5 units of Taq DNA polymerase. The amounts of PCR primers in each tube were as follows: tube 1, 100 pmoles oligo 1, 100 pmoles oligo 2; tube 2, 75 pmoles oligo 1, 100 pmoles oligo 2; tube 3, 50 pmoles oligo 1, 100 pmoles oligo 2; tube 4, 25 pmoles oligo 1, 100 pmoles oligo 2; tube 5, 10 pmoles oligo 1, 100 pmoles oligo 2; tube 6, 100 pmoles primer mix 1; tube 7, 200 pmoles primer mix 1.

100 microlitres of Nujol oil was placed on top of each solution and the tubes were incubated in a thermal cycler with the following heating protocol: 60°C for 1 minute, 72°C for 2 minutes, 94°C for 1 minute, and this cycle was repeated 40 times. 20 microlitres from each tube was mixed with 2 microlitres of 50% glycerol containing 0.1% bromophenol blue, 0.1% xylene cyanol and this mixture was loaded onto a 1% agarose gel containing ethidium bromide (0.5 microgrammes/ml). Also included in the gel were O x 174 size markers. The presence of the expected 177 bp PCR product is clearly visible in lanes 6 and 7, as well as in lanes 1-5, thus demonstrating that the PCR works using a pair of primers prepared according to the method of the invention.

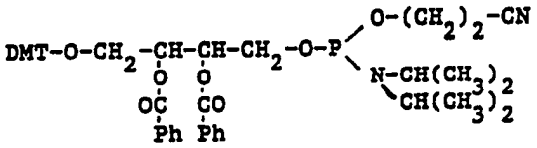
Figure 1

Electrophoretic analysis of PCR products described above. Lanes 1-7 contain samples from tubes 1-7 described above, respectively. Lane M, O x 174 size markers.

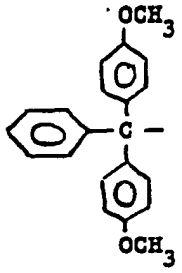
EXAMPLE 4
Preparation of Reagent M2:
1-O-(4,4'-Dimethoxytrityl) -2,3-di-O-benzoyl-4- O-(2-cyanoethyl -N,N-diisopropyl)phosphoroamiditothreitol.

This was synthesized using the preparations numbered 1 to 5 described below.

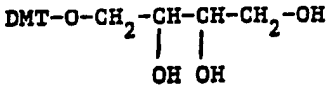
The structure of reagent M2 is as follows:



wherein DMT is:



Step 1) Preparation of 1-O-(4,4'-dimethoxytrityl) threitol.

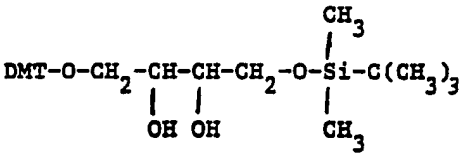


To a solution of threitol (Aldrich, 6.1g, 50 mmol) in dry pyridine (Aldrich, 200ml) at room temperature was added 4,4' dimethoxytrityl chloride (Courtaulds, 17g, 50 mmol) with stirring. When dissolution was complete, 4-(N,N-dimethylamino)pyridine (Aldrich, 100mg) was added. The solution was stirred at room temperature overnight. The solvent was removed by rotary evaporation and residual pyridine was removed by repeated co-evaporation with toluene (BDH). The residue was redissolved in dichloromethane (BDH), and washed three times with an equal volume of saturated sodium bicarbonate solution (Aldrich). The organic s luti n

was dried by the addition of anhydr us sodium sulphate (Interchem, UK) and filtered. The filtrat was evaporated to a gum, redissolved in the minimum v lume f dichloromethane:methanol (19:1) and applied t a silica column (Merck 7734). Elution with the same solvent gave the title compound as a colourless gum (7g, 33%).

^{1}H NMR: δ ($CDCl_3$): 3.4-3.2, 2H, two double doublets, CH_2O-DMT; 3.7-3.6, 2H, complex multiplet, CH_2OH; 3.85-3.75, 8H, complex multiplet, 2x $-OCH_3$ and 2x C-H; 6.84-6.81, 4H, complex multiplet, aromatics; 7.42-7.25, 9H, complex multiplet, aromatics.

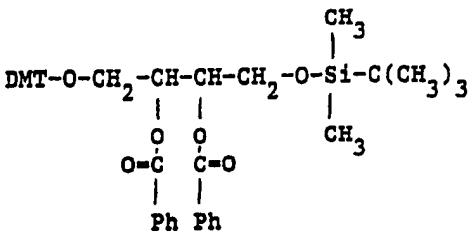
Step 2) Preparation of 1-O-(4,4'-dimethoxytrityl)-4-O-(tert.)-butyldimethylsilyl threitol.



To a solution of the product from step 1), (7g, 16.5 mmol) in dry pyridine (100ml) was added tert.butyl dimethylsilyl chloride (Aldrich, 2.7g, 18.15 mmol) with stirring. When dissolution was complete, 4-(N,N-dimethylamino) pyridine (100mg) was added, and the solution was stirred at room temperature overnight when TLC in dichloromethane: methanol (19:1) showed there to be no starting material present. The solvent was evaporated under reduced pressure, and residual pyridine was removed by repeated co-evaporation with toluene. The residue was redissolved in dichloromethane (300ml) and this solution was washed three times with an equal volume of saturated sodium bicarbonate solution, dried (sodium sulphate), filtered and evaporated under reduced pressure to give a gum which was dissolved in the minimum volume of dichloromethane:methanol (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless gum (6.5g, 73%).

^{1}H NMR: δ ($CDCl_3$): -0.042, 6H, two singlets, 2x CH_3-Si; 0.8, 9H, singlet, $(CH_3)_3$-C-Si; 3.3-3.1, 2H, two double doublets, CH_2-O-DMT; 3.7-3.55, 2H, two double doublets, CH_2-O-Si; 3.8, 8H, complex multiplet, 2x OCH_3 and 2x C-H; 6.8, 4H, complex multiplet, aromatics; 7.3-7.1, 9H complex multiplet, aromatics.

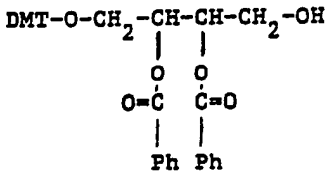
Step 3) Preparation of 1-O-(4,4'-dimethoxytrityl) -2,3,-di-O-benzoyl -1-O (tert.)butyldimethylsilyl threitol.



To a solution of the product from the step 2) (2.2g, 4.125mmol) in dry pyridine (100ml) was added benzoyl chloride (Aldrich, 1.05ml, 9.075mmol) dropwise with stirring. The solution was stirred at room temperature for 3 hours, when TLC in dichloromethane showed there to be no starting material present. The solvent was removed by evaporation under reduced pressure, and residual pyridine was removed by repeated co-evaporation with toluene. The residue was redissolved in dichloromethane and this solution was washed with three equal volumes of saturated sodium bicarbonate solution, dried (sodium sulphate), filtered and evaporated under reduced pressure to a gum which was redissolved in the minimum volume of dichloromethane and applied to a silica column. Elution with the same solvent gave the title compound as a white foam (2.7g, 87.6%).

^{1}H NMR: δ ($CDCl_3$): 0.0- (-)0.1, 6H, multiple singlets, 2x CH_3-Si; 0.8-0.9, 9H, multiple singlets, $(CH_3)_3$-C-Si; 3.65, 3H, singlet, OCH_3; 3.75, 3H, singlet, OCH_3; 4.1-3.85, 4H, complex multiplet, CH_2-O-DMT and CH_2-O-Si; 5.75-5.45, 2H, complex multiplet; 2x C-H; 6.65-6.55, 2H, complex multiplet, aromatics; 6.85-6.75, 2H, complex multiplet, aromatics; 7.3-7.05, 9H, complex multiplet, aromatics; 7.6-7.35, 6H, complex multiplet, aromatics; 8.1-7.8, 4H, complex multiplet, aromatics.

Step 4) Preparation of 1-O-(4,4'-dimethoxytrityl)-2,3- di-O-benzoyl threitol.



The product from step 3) (2.7g, 3.6mmol) was dissolved in a mixture f tetrahydrofuran (Aldrich), pyridine and water (100ml, 8:1:1 respectively) and a solution of tetrabutyl ammonium fluoride in tetrahydrofuran (Aldrich, 1M, 15ml) was added. The solution was kept at room temperature for 3 days and then evaporated under reduced presuure to an oil which was redissolved in 100ml of dichloromethane. This solution was washed four times with an equal volume of water and once with an equal volume of saturated sodium chloride solution, dried (sodium sulphate), filtered and evaporated under reduced pressure to a gum. This was redissolved in the minimum volume of dichloromethane: methanol (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a white foam (1.2g, 52.7%).

^{1}H NMR: δ ($CDCl_3$): 3.6-3.35, 2H, complex multiplet, CH_2-OH; 3.8-3.7, 6H, multiple singlets, 2x OCH_3; 4.55-4.35, 2H, complex multiplet, CH_2-O-DMT; 4.72-4.6, 1H, complex multiplet, C-H; 5.6-5.35, 1H complex multiplet, C-H; 6.85-6.7, 4H, complex multiplet, aromatics; 7.6-7.1, 15H, complex multiplet, aromatics; 8.1-7.85, 4H, complex multiplet, aromatics.

Step 5) Preparation of Reagent M2

To a solution of the product from step 4) (1.2g, 1.9mmol) in dry dichloromethane (50ml) was added dry (by distillation from calcium hydride) diisopropylethylamine (1.4ml, 25mmol) by syringe transfer under a stream of dry argon (Air Products). To this stirred solution was added (also by syringe) 2-cyanoethyl-N,N-diisopropylamino chlorophosphine (Aldrich, 0.51ml, 2.3mmol), dropwise with stirring. The solution was stirred at room temperature under a stream of dry argon for 30 minutes when TLC in dichloromethane:triethylamine (19:1) showed there to be no starting material present. Dry methanol (5ml) was added and the solution was diluted with 200ml of ethyl acetate (BDH). This solution was washed with three equal volumes of saturated sodium chloride solution and one volume of water. The organic layer was separated, dried (sodium sulphate), filtered and evaporated to a gum which was dissolved in the minimum volume of dichloromethane: hexane: triethylamine (42:55:3) and applied to a silica column. Elution with the same solvent followed by elution with dichloromethane: triethylamine (19:1) gave the title compound as a colourless gum (0.6g, 38%).

^{1}H NMR: δ ($CDCl_3$): 1.3-1.0, 14H, complex multiplet, 2x $(CH_3)_2CH$-; 2.5, 2H, pseudo triplet, CH_2CN; 3.8-3.4, 10H, complex multiplet, 2x OCH_3, CH_2-O-DMT and CH_2-O-P; 4.7-4.45, 3H, complex multiplet, CH_2-O-P and C-H; 5.8-5.55, 1H, complex multiplet, C-H; 6.8-6.6, 4H, complex multiplet, aromatics; 7.6-7.1, 15H, complex multiplet, aromatics; 8.1-7.8, 4H, complex multiplet, aromatics.

EXAMPLE 5
Preparation of two oligonucleotides on a single support.

An oligonucleotide was formed on a solid support via a first (conventional) cleavable link using the protocol supplied with the Applied Biosystems 380B DNA synthesizer, using the 3'-(2-cyanoethyl-N,N-diisopropylaminophosphoramidites of 5'-dimethoxytrityl-N^4-benzoyl-2'deoxycytidine, 5'-dimethoxytrityl-N^2-isobutyryl-2'deoxyguanosine, 5'dimethoxytrityl-N^6-benzoyl-2'-deoxyadenosine and 5'-dimethoxytritylthymidine (Cruachem) as the precursors of the individual nucleotides. A cleavable linker moiety was attached to the first oligonucleotide by means of reagent M2. Reagent M2 was dissolved in anhydrous acetonitrile to a concentration of 0.1M, and a bottle containing this solution was attached to one of the spare reagent ports on the DNA synthesizer. A column containing controlled pore glass bearing the 5'-protected nucleoside (in this case deoxyadenosine) connected by means of a (conventional) cleavable linker succinylglycylglycylaminopropyl (Cruachem) was attached to the synthesiser. The synthesiser was then programmed to synthesise the following sequence:

(5') CTATTCAAAATCGGAGCTCTAAGAT-L'-TAGGGATTTGATTTTACGA (3')

(whereby the cleavable linker moiety L' is introduced by means of reagent M2), using standard synthesis cycles employed on the Applied Biosystems 380B DNA synthesizer. The duration of the reaction steps and the volume of reagents used for coupling, oxidation, capping and detritylation were identical for each coupling, including that of reagent M2. The synthesiser was programmed to perform the conventional concentrated ammonia wash of the column to release the oligonucleotides into collection vials.

In this manner the synthesiser achieves the steps of a) forming a first oligonucleotide of sequence (5'-3') TAGGGATTTGATTTTACGA by succesive reaction of the nucleoside precursors with connected to the controlled pore glass support via the 3'-OH group and a (conventional) first cleavable link, b) attaching to the first oliognucleotide a cleavable linker moiety by means of reagent M2, and c) forming a second oligonucleotide was formed on the cleavable linker moiety having the sequence (5'-3') CTATTCAAAATCGGAGCTAAGAT, to give two oligonucleotides separated by a cleavable linker moiety and bound to a solid support by a cleavable link, as illustrated by the formula:

5' 3' 5' 3'
d(CTATTCAAAATCGGAGCTCTAAGAT)-L'-d(TAGGGATTTGATTTTACGA)-X-support

wherein -L'- is a cleavable linker of formula $-CH_2-CH(O\text{-benzoyl})-CH(O\text{-benzoyl})-CH_2-$, attached to the 5' and 3' oxygen of the first and second oligonucleotides respectively by a group of formula $-P(O)(OCH_2CH_2CN)-O-$, and X is a first cleavable link contained in the succinylglycylglycyl-aminopropyl spacer. The synthesiser also

performs the cleavage of the first cleavable link X by the ammonia treatment as in step d) in the method of the invention.

The eluted oligonucleotide in the ammonia solution was incubated at 55°C for 16 hours and evaporated to dryness under reduced pressure. The residue was redissolved in 1ml of water, and 100µl of this solution were mixed with 100µl of piperidine and incubated at 55°C for between 16 and 72 hours.

In this manner the cleavable link L' is cleaved as described in step d) of the method of the invention.

Three other oligonucleotides were also synthesized by conventional procedures as described above but omitting the treatment with piperidine. These were designed to represent control molecules to be used in the analysis of products generated by the piperidine treatment above. These oligonucleotides had the following sequences:

1) (5') CTATTCAAAATCGGAGCTCTAAGATTAGGGATTTGATTTTACGA (3')
2) (5') CTATTCAAAATCGGAGCTCTAAGAT (3')
3) (5') TAGGGATTTGATTTTACGA (3')

Thus, oligonucleotides 2) and 3) are of identical length and sequence to the products expected from cleavage of the oligonucleotide containing the cleavable link.

The presence of hydroxyl groups at the 3' and 5' ends of the oligonucleotides so produced was determined by the incorporation of a radioactive phosphorus at these positions as described below.

Incorporation of radiolabelled phosphate at the 5' end of oligonucleotides.

After treatment of the oligonucleotides with either concentrated ammonium hydroxide or 50% piperidine the solutions were lyophilized and the oligonucleotides were redissolved in water to a concentration of approximately 1 mg/ml. One microlitre of this solution was then added to an Eppendorf tube containing water (6µl), 10x reaction buffer ("One Phor All", Pharmacia, 1µl), [gamma ^{32}P] adenosine triphosphate (Amersham, 1µl) and T4 Polynucleotide kinase (Pharmacia, 1µl). This mixture was then incubated at 37°C for one hour. Ethanol (30µl) was added, the contents were mixed by repeated inversion of the tube, and the sample was incubated at -70°C for 15 minutes. The tube was spun in an Eppendorf centrifuge (Model 5415) at 14000 rpm for 15 minutes, and the supernatant was discarded. The pellet was dried briefly *in vacuo* and was redissolved in 10µl of a solution containing 80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 10µM EDTA. This solution was loaded into one of the wells of a denaturing polyacrylamide gel (8% acrylamide, 50% urea) adjacent to the appropriate radiolabelled size markers, and the gel was run at 40W for approximately two hours. The locations and sizes of labelled DNA fragments were determined by autoradiography.

The presence of strong bands co-migrating with bands due t olig nucle tides of 25 residues (25 phosphates) and 19 residues (19 phosphat s) indicated that scission f the cleavable link had ccurred, thus generating the desired products.

Inco oration of radiolabelled phosphate at the 3'- end of oligonucleotide.

One microlitre of the solution of oligonucleotide described above was added to an Eppendorf tube containing water (5µl), 5x reaction buffer ("TdT Tailing Buffer", BRL, 2µl), [alpha ^{32}P] 2'-deoxyadenosine triphosphate (Amersham, 1µl) and terminal deoxynucleotidyl transferase (BRL, 1µl). This mixture was then incubated at 37°C for one hour. Radiolabelled DNA was recovered by precipitation from ethanol and then analyzed by denaturing gel electrophoresis exactly as described above for 5'- end-labelled fragments.

The presence of strong bands co-migrating with bands due to oligonucleotides of 26 residues (25 phosphates) and 20 residues (19 phosphates) indicated that scission of the cleavable link had occurred, thus generating the desired products.

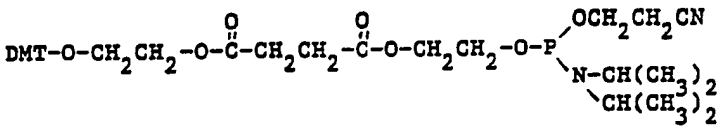
The mixture of oligonucleotides produced in step (d) described above was analyzed by reverse-phase HPLC on a Waters µBondapak C18 column using a linear gradient from 0-30% buffer B in buffer A over 45 minutes where buffer A was 0.1M triethylammonium acetate (pH 7.5) and buffer B was 80% acetonitrile in 0.1M triethylammonium acetate (pH 7.5).
A control oligonucleotide of 19 residues (18 phosphates) had a retention time of 28 minutes, an oligonucleotide of 25 residues (24 phosphates) had a retention time of 31 minutes and an oligonucleotide of 44 residues (43 phosphates) had a retention time of 34 minutes under these conditions.

The HPLC profile of the mixture of oligonucleotides produced in step (d) above showed peaks corresponding (within experimental error) to the presence of oligonucleotides of 19 and 25 residues (18 and 24 phosphates respectively) thus confirming that scission of the cleavable link had occurred generating the desired products.

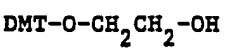
EXAMPLE 6
Preparation of reagent M3: 1-O-(4,4'-dimethoxytrityl)-1,2-dihydroxyethan-2-yl-1-(1,4-dicarboxy) butanoate-4-(1,2-dihydroxy-2-(2-cyanoethyl-N,N-diisopropylphosphoramidite) ethan-1-yl ester.

This was prepared using the preparations numbered 1 to 4 described below.

The structure of Reagent M3 is as follows:



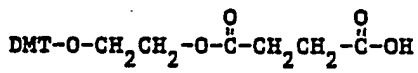
Step 1) Preparation of 1-O-(4,4'-dimethoxytrityl) -1,2-dihydroxyethane.



To a solution of 1,2-dihydroxyethane (Aldrich, 6.2g, 100mmol) in dry pyridine (200ml) was added 4,4'-dimethoxytrityl chloride (33.8g, 100mmol) with stirring. When dissolution was complete, 4-(N,N-dimethylamino)pyridine (200mg) was added. The solution was stirred at room temperature overnight. The solvent was then removed under reduced pressure and residual pyridine was removed by repeated co-evaporation with toluene. The residue was redissolved in dichloromethane (300ml) and washed with three equal volumes of saturated sodium bicarbonate solution. The organic layer was separated, dried (sodium sulphate), filtered and evaporated under reduced pressure to a gum which was redissolved in dichloromethane: methanol (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless gum (9g, 25%).

^{1}H NMR: δ ($CDCl_3$): 3.3, 2H, pseudo triplet, CH_2-O-DMT; 3.8, 8H, multiplet, 2x OCH_3 and CH_2OH; 6.85-6.75, 4H, multiplet, aromatics; 7.4-7.1, 9H, complex multiplet, aromatics.

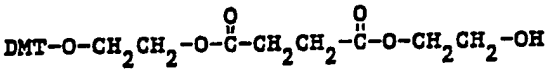
Step 2) Preparation of 1-O-(4,4'-dimethoxytrityl)-1,2-dihydroxyethan-2-yl-1-(1,4-dicarboxy)butanoate



The product from step 1) (9g, 24.7mmol) was dissolved in dry pyridine (100ml) and succinic anhydride (Aldrich, 2.72g, 27.2mmol) was added. When dissolution was complete, 4-(N,N-dimethylamino) pyridine (50mg) was added and the solution was stirred at room temperature overnight. The solvent was evaporated under reduced pressure and residual pyridine was removed by repeated co-evaporation with toluene. The residue was dissolved in dichloromethane (300ml) and this solution was washed with three equal volumes of ice-cold 10% citric acid and one volume of water. The organic layer was separated, dried (sodium sulphate), filtered and evaporated under reduced pressure to a gum which was redissolved in the minimum volume of dichloromethane: methanol (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless gum (9g, 78.5%).

^{1}H NMR: δ($CDCl_3$): 2.7, 4H, singlet, 2x CH_2CO; 3.25, 2H, triplet, CH_2-O-DMT; 3.8, 6H, singlet, 2x $-OCH_3$; 4.25, 2H, complex multiplet, CH_2OCO; 6.85-6.75, 4H, complex multiplet, aromatics; 7.45-7.1, 9H, complex multiplet, aromatics.

Step 3) Preparation of 1-O-(4,4'-dimethoxytrityl)-1,2-dihydroxyethan -2-yl -1-(1,4-dicarboxy)butanoate-4-(1,2-dihydroxy)ethan-1-yl ester.



The product from step 2) (8g, 17.24mmol) was dissolved in dry pyridine (200ml) containing 1,2-dihydroxyethane (6.2g, 100mmol). To this solution was added 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (Aldrich, 3.75g, 19.5mmol). The solution was stirred at room temperature overnight when TLC in dichloromethane: methanol (19:1) showed there to be no starting material present. The solvent was removed under reduced pressure and residual pyridine was removed by repeated co-evaporation with toluene. The residue was redissolved in ethyl acetate and washed with three equal volumes of saturated sodium chloride solution and one volume of water. The organic layer was separated, dried (sodium sulphate), filtered and evaporated under reduced pressure to a gum which was redissolved in the minimum volume of dichloromethane: methanol (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless gum (2g, 22.6%).

^{1}H NMR: δ ($CDCl_3$): 2.7, 4H, singlet, 2x CH_2CO; 3.25, 2H, pseudo triplet, CH_2-O-DMT; 3.85-3.7, 8H, complex multiplet, 2x $-OCH_3$ and CH_2OH; 4.3-4.2, 4H, complex multiplet, 2x CH_2OCO; 6.85-6.75, 4H, complex multiplet, aromatics; 7.4-7.1, 9H, complex multiplet, aromatics.

Step 4) Preparation of Reagent M3

The product from step 3) (2g, 3.9mmol) was dissolved in dry dichloromethane (50ml) and the solution was stirred under a stream of dry argon. To this solution was added dry diisopropylethylamine (2.7ml, 16mmol) and 2-cyanoethyl-N,N-diisopropylaminochlorophosphine (1.05ml, 4.72mmol). The solution was stirred at room temperature under a stream of dry argon for 30 minutes when TLC in dichloromethane: methanol (19:1) showed there to be no starting material present. The reaction was quenched by addition of dry methanol (5ml) and the solution was diluted with ethyl acetate (200ml). This solution was washed with three equal volumes f saturated sodium chloride solution, and one volume of water. The organic layer was separated, dried (sodium sulphate), filtered and

evap rated under reduced pressure to a gum which was redissolved in th minimum volume of dichloromethane:triethylamine (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless gum (1.8g, 65%).

^{1}H NMR:δ($CDCl_3$): 1.25-1.1, 14H, complex multiplet, $(CH_3)_2CH$ x2; 2.6, 2H, triplet, CH_2CN; 2.65, 4H, singlet, 2x CH_2CO; 3.25, 2H, triplet, CH_2-O-DMT; 3.7-3.5, 4H complex multiplet, 2x $-CH_2-OP$; 3.8, 6H, singlet, 2x $-OCH_3$; 4.25, 4H, pseudo triplet, 2x CH_2OCO; 6.85-6.75, 4H, complex multiplet, aromatics; 7.4-7.15, 9H, complex multiplet, aromatics.

EXAMPLE 7:

The method of example 5) was repeated to synthesise two oligodeoxyribonucleotides bound to a solid support except that reagent M3 was used, dissolved in anhydrous acetonitrile to a concentration of 0.15M, in place of reagent M2.

The two oligonucleotides bound to a solid support by a cleavable link are illustrated by the formula given in Example 5, step c, wherein L' is a cleavable linker moiety of formula:

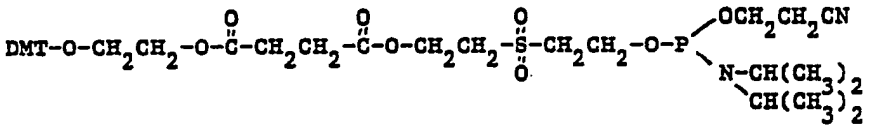
$$-CH_2CH_2OCOCH_2CH_2COOCH_2CH_2-$$

The two oligonucleotides found after step (d) were analysed as described in Example 5 and found to be identical by electrophoresis and HPLC to those described in Example 5, demonstrating scission of the cleavable link.

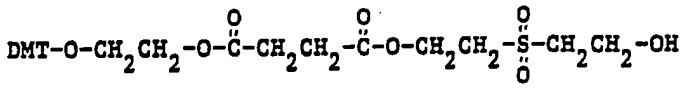
EXAMPLE 8:
Preparation of reagent M4:
1-O-(4,4'-Dimethoxytrityl)-1,2-dihydroxyethan-2-yl -1-(2-[{2-cyanoethoxy)-N,N-(diisopropylamino)phosphanyloxy] ethylsulfonyl)ethyl succinate.

This was synthesised using the preparations numbered 1 to 2 described below.

The structure of Reagent M4 is as follows:



Step 1) Preparation of 1-O-(4,4'-dimethoxytrityl)-1,2-dihydroxyethan-2yl 2-(2-hydroxyethylsulfonyl)ethyl succinate.



To a solution of 1-O-(4,4'-dimethoxytrityl) -1,2- dihydroxyethan -2-yl -1-(1,4-dicarboxy)butanoate prepared as described in example 6.2 above (1.74g) in dichloromethane (20ml) was added 1,3-N,N-dicyclohexylcarbodiimide (383mg, 0.5meq) and the mixture was stirred at room temperature for 45 minutes. Dicyclohexylurea was filtered off and washed with dichloromethane (4ml). The filtrate and washings were combined and evaporated under reduced pressure to a yellow oil which was redissolved in dry pyridine (15ml). To this was added sulphonyldiethanol (1.54g, 2.7meq; prepared by toluene azeotropic dehydration of 65% aqueous material supplied by Aldrich) in dry pyridine (5ml). The solution was stirred at room temperature for 23 hours and evaporated under reduced pressure. Residual pyridine was removed by repeated co-evaporation with toluene. The residual oil was redissolved in dichloromethane:methanol (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a yellow glass (543mg, 48.4%)

^{1}H NMR: δ ($CDCl_3$): 2.7, 4H, complex multiplet, 2x CH_2COO; 3.22, 3H, pseudo triplet, CH_2-O-DMT and -OH; 3.3, 2H, triplet, CH_2OH; 3.43, 2H, triplet, CH_2SO_2; 3.76, 6H, singlet, 2x $-OCH_3$; 4.03, 2H, triplet, CH_2SO_2; 4.27, 2H, triplet, CH_2OCO; 4.53, 2H, triplet, CH_2OCO; 6.8, 4H, multiplet, aromatics; 7.3, 9H, complex multiplet, aromatics.

Step 2) Preparation of Reagent M4

The product from step 1) (0.54g, 0.9mmol) was dissolved in dry dichloromethane (50ml) and the solution was stirred under a stream of dry argon. To this solution was added dry diisopropylethylamine (0.61ml, 3.6mmol) and 2-cyanoethyl-N,N-diisopropylaminochlorophosphine (0.24ml, 1.08mmol). The solution was stirred at room temperature under a stream of dry argon for 30 minutes when TLC in dichloromethane: methanol (19:1) showed there to be no starting material present. The reaction was quenched by addition of dry methanol (5ml) and the solution was diluted with ethyl acetate (200ml). This solution was washed with three equal volumes of saturated sodium chloride solution, and one volume of water. The organic layer was separated, dried (sodium sulphate), filtered and evaporated under reduced pressure to a gum which was redissolved in the minimum volume of dichloromethane:triethylamine (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless gum (0.48g, 66.6%).

^{1}H NMR: δ ($CDCl_3$): 1.31-1.17, 14H, multiplet, $(CH_3)_2CH$ x2; 2.74-2.58, 6H, complex multiplet, 2x $-CH_2COO$ and CH_2CN; 3.33-3.25, 2H, CH_2-ODMT; 3.45, 2H, multiplet, CH_2SO_2; 3.65-3.51, 4H, complex multiplet, 2x $-CH_2OP$; 3.81, 6H, singlet, 2x OCH_3; 4.15-4.05, 2H, multiplet, CH_2SO_2; 4.25, 2H, pseudo triplet, CH_2OCO, 6.84-6.80, 4H, multiplet, aromatics; 7.45-7.14, 9H, complex multiplet, aromatics.

EXAMPLE 9

The method of Example 5 was repeated to synthesise two oligonucleotides bound to a solid support, except that reagent M4 was dissolved in anhydrous acetonitrile to a concentration of 0.15M and was used in place of reagent M2.

The two oligonucleotides bound to a solid support by a cleavable link are illustrated by the formula given in Example 5, step c, wherein L' is a cleavable linker moiety of formula:

$$-CH_2CH_2OCOCH_2CH_2COOCH_2CH_2SO_2CH_2CH_2-$$

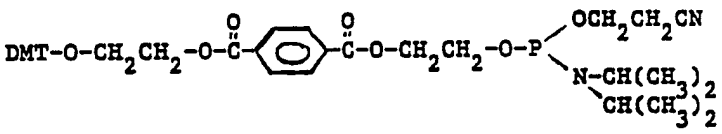
The two oligonucleotides found after step (d) were analysed as described in example 5 and found to be identical to those described in Example 5, demonstrating scission of the cleavable link.

EXAMPLE 10

Preparation of reagent M5; 1-O-(4,4'-dimethoxytrityl)-1,2-dihydroxyethan-2-yl-1-(1,4-dicarboxy) benzoate-4-(1,2-dihydroxy-2-(2-cyanoethyl- N,N-diisopropylphosphoramidite) ethan-1-yl ester.

This was synthesized using the preparations numbered 1 to 3 described below.

The structure of reagent M5 is as follows:

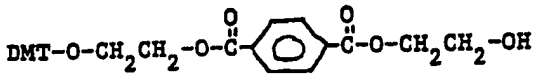


Step 1) Preparation of 1,4-bis-(1-O-[4,4'-dimethoxytrityl]-1,2-dihydroxyethan-2-yl)-benzene-1,4-dicarboxylate.

To a stirred solution f 1-O-(4,4'-dimeth xytrityl)-1,2-dihydroxyethane (prepared as described in example 6, step 1 above) (ca 10g, 27mmol) in dry pyridine (70ml) was added solid terephthalic chloride (1.83g, 9 mmole) and 4-N,N-dimethylaminopyridine (50mg). The suspension was stirred at room temperature overnight after which most of the solid material was observed to have dissolved. The solvent was then removed under reduced pressure. The residue was redissolved in dichloromethane (200ml) and washed with three equal volumes of saturated sodium bicarbonate solution. The organic layer was separated, dried (sodium sulphate), filtered and evaporated under reduced pressure. Remaining pyridine was removed by co-evaporation with toluene to give a gum which was redissolved in dichloromethane: methanol (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless gum (2.74g, 35%).

^{1}H NMR: δ ($CDCl_3$): 3.43, 4H, triplet, 2x $-CH_2-ODMT$; 3.79-3.77, 12H, two singlets, 4x $-OCH_3$; 4.52, 4H, triplet, 2x $-CH_2OCO$; 6.83-6.76, 8H, complex multiplet, aromatics; 7.49-7.13, 18H, complex multiplet, aromatics; 8.19, 4H, singlet, aromatics.

Step 2) Preparation of 1-O-(4,4'-Dimethoxytrityl) -1,2-dihydroxyethan -2-yl -1-(1,4-dicarboxy)benzoate-4-(1,3-dihydroxy)ethan-1-yl ester.



The product from step 1 (2.7g, 3.5mmol) was dissolved in dry dichloromethane (50ml) and a solution of trichloroacetic acid in dichloromethane (Cruachem, 3% TCA, 76ml) was added. The solution was stirred at room temperature when TLC showed there to be a mixture of products present. The solution was washed with saturated sodium bicarbonate solution (4x200ml), dried (sodium sulphate), filtered and evaporated to an oil which was redissolved in dichloromethane : methanol (19:1) and applied to a silica column. Elution with the same solvent gave 300 mg of a compound which ran more slowly than the starting material on TLC. (Further treatment of a sample of this material with TCA converted it into a more polar material with concomitant formation of a deep orange colour, thus indicating the presence of a dimethoxytrityl group). This product was used in the next step without further characterization.

Step 3) Preparation of Reagent M5.

The product from step 2 (0.3g, 0.54mmol) was dissolved in dry dichloromethane (50ml) and the solution was stirred under a stream of dry arg n. To this solution was added dry diisopropylethylamine (.37ml, 2.16mmol) and 2-cyan ethyl-N,N-diisopropylaminochlorophosphine (0.15ml, 0.65mmol). Th s luti n was stirred at ro m temperature under a

stream of dry argon for 30 minutes when TLC in dichlor methane: methanol (19:1) sh wed there t be n starting material present. The reaction was quenched by additi n f dry methanol (5ml) and the solution was diluted with ethyl acetate (200ml). This solution was washed with three equal volumes of saturated sodium chloride solution, and one volume of water. The organic layer was separated, dried (sodium sulphate), filtered and evaporated under reduced pressure to a gum which was redissolved in the minimum volume of dichloromethane:triethylamine (19:1) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless gum (0.37g, 90%).

^{1}H NMR: δ ($CDCl_3$): 1.26-1.15, 14H, complex multiplet, $(CH_3)_2CH$ x2; 2.6, 2H, triplet, CH_2CN; 3.45, 2H, triplet, CH_2-ODMT; 3.72-3.52, 4H, complex multiplet, 2x $-CH_2-OP$; 3.79, 6H, singlet, 2x $-OCH_3$; 4.52, 4H, triplet, 2x $-CH_2-OCO$; 6.8, 4H, multiplet, aromatics; 7.49-7.19, 9H, complex multiplet, aromatics; 8.16, 4H, singlet, aromatics.

EXAMPLE 11.

The method of Example 5 was repeated to synthesise two oligodeoxyribonucleotides bound to a solid support, except that in place of reagent M2 there was used a 0.13M solution of reagent M5 in anhydrous acetonitrile.

The two oligonucleotides bound to a solid support by a cleavable link are illustrated by the formula given in Example 5, step c, wherein L' is a cleavable linker moiety of formula:

$$-CH_2CH_2OCO(C_6H_4)COOCH_2CH_2-$$

The two oligonucleotides found after step (d) were analysed as described in Example 5 and found to be identical to those described in Example 5, demonstrating scission of the cleavable link.

Claims

1. A method for the synthesis of a plurality of oligonucleotides in which an oligonucleotide is formed by sequential reactions of precursors of individual nucleotides on a support, comprising the steps of (a) forming a first oligonucleotide; (b) attaching to said first oligonucleotide a cleavable linker moiety; (c) forming a second oligonucleotide on the cleavable linker moiety; and (d) cleaving the linker moiety to give the desired oligonucleotides.

2. A method according to Claim 1 wherein the cleavable linker moiety is attached to the first oligonucleotide by means of a reagent which is capable of connecting to said first oligonucleotide and upon which a second oligonucleotide may be formed, and which can be broken to separate the first and second oligonucleotides under conditions which do not significantly affect the oligonucleotides.

3. A method for the synthesis of a plurality of oligonucleotides comprising the steps of:
   (a) forming a first oligonucleotide on a first cleavable link attached to a solid support;
   (b) attaching to the first oligonucleotide a cleavable linker moiety;
   (c) forming a second oligonucleotide on the cleavable linker moiety; and
   (d) cleaving the first cleavable link and the cleavable linker moiety to give a plurality of oligonucleotides.

4. A method according to Claim 1 or Claim 3 wherein after step (d) has been performed, organic residues of the cleaved linker moiety do not remain attached to the oligonucleotides.

5. A method according to Claim 1 or Claim 3 which does not contain a step in which hybridisation of the first or second oligonucleotide with a further oligonucleotide is attempted.

6. A method according to Claim 1 or Claim 3 wherein cleavage of the cleavable linker moiety results in a plurality of oligonucleotides each having at the 3' and 5' position a group selected from hydroxy and phosphate.

7. A method according to Claim 1 or Claim 3 wherein the cleavable linker moiety is attached by means of a modified nucleoside.

8. A method according to Claim 1 or Claim 3 wherein steps (b) and (c) are repeated from 1 to 100 times.

9. A modified nucleoside of Formula II:

Z-Nuc-L'-O-PA (II)

wherein:

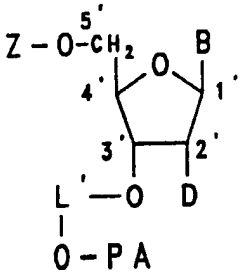
Nuc is a nucleoside in which the base optionally is protected;

Z is a protecting group attached to the 5' oxygen of Nuc;

-O-PA is a phosphoramidite group, a phosphate ester group, a H-phosphonate group or other group capable of conversion to a phosphodiester group; and

L' is a cleavable linker moiety.

10. A modified nucleoside of the Formula (III):



(III)

wherein;

Z, L' and -O-PA are as defined in Claim 9;

B is an optionally protected base; and

D is H or a protected hydroxyl group.

11. A modified nucleoside according to Claim 9 or Claim 10 wherein L' is of formula:

-(i)-(ii)-(iii)-

wherein;

(i) is carbonyl, -CONH- or $-C(=NH_2^+)-$;

(ii) is a spacer group; and

(iii) is a group capable of giving rise to beta-elimination of a phosphate ester group, or a group of formula $R_9-CR_7(OZ^2)-CR_7R_8-$ or $-CO.-O-CR_7R_{11}-CR_7R_{10}-$.

wherein;

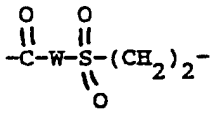
each R_7 independently is H or C_{1-4}-alkyl;

one of R_8 & R_9 is a single bond and the other is H or C_{1-4}-alkyl;

R_{10} & R_{11} are each independently H or C_{1-4}-alkyl or R_{10} together with R_{11} and the carbon atoms to which they are attached form an optionally substituted 4,5,6 or 7 membered alicyclic or heterocyclic ring; and

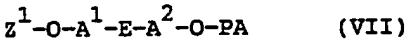
Z^2 is a protecting group.

12. A modified nucleoside according to Claim 9 or Claim 10 wherein L' is of the formula:



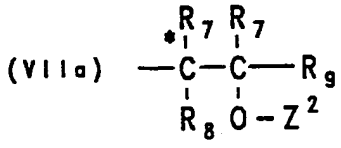
wherein W is a divalent organic spacer group.

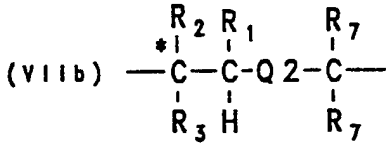
13. A compound of Formula (VII):

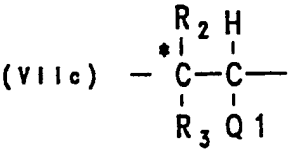


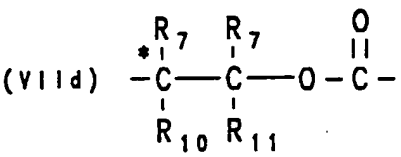
wherein;

A^1 and A^2 are each independently of the formula (VIIa), (VIIb), (VIIc) or (VIId) wherein the carbon atom marked with an asterisk is attached to the oxygen atom shown in Formula (VII):









Z^1 is a protecting group;

R_1, R_2 & R_3, are each independently H or alkyl;

Q1 is an electron withdrawing group;

Q2 is $-SO_2-$;

-O-PA is a phosphoramidite group, a phosphate ester group, or a H-phosphonate group;

each R_7 independently is H or C_{1-4}-alkyl;

one of R_8 and R_9 is a single bond by means of which the group of formula (VIIa) is attached to E, and the other is H or C_{1-4}-alkyl;

Z^2 is a protecting group;

R_{10} & R_{11} are each independently H or C_{1-4}-alkyl or R_{10} together with R_{11} and the carbon atoms to which they are attached form an optionally substituted 4, 5, 6 or 7 membered alicyclic or heterocyclic ring;

E is a single covalent bond or a spacer group; and

provided that when A^1 and A^2 are both of Formula (VIId) E is a spacer group.

14. A compound according to Claim 13 wherein E is an optionally substituted alkyl, alicyclic or aryl spacer group.

15. A compound according to Claim 13 wherein E is phenylene or an alkyl group containing up to 6 carbon atoms.

16. A compound according to Claim 13 wherein A^1 and A^2 are each independently selected from (VIIa), (VIIb) and (VIId) wherein the carbon atom marked with an asterisk is attached to the oxygen shown in Formula (VII).

17. A compound comprising two or more oligonucleotides linked by a group or groups containing a cleavable linker moiety of formula $-A^1-E-A^2-$ or -L'- wherein A^1, E and A^2 are as defined in Claim 13 and L' is as defined in Claim 9.

18. A solid support, suitable for use in an automated oligonucleotide synthesiser, of Formula (VIII) or (IX):

$SUP-P^1-L'-Nuc-Z$ (VIII)

$SUP-P^1-A^2-E-A^1-O-Z^1$ (IX)

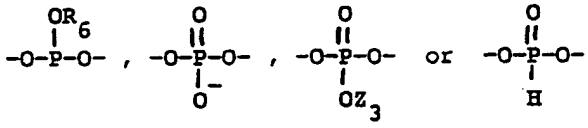
wherein;

SUP is a solid support;

L', Nuc and Z are as defined in Claim 9;

A^2, E, A^1 and Z^1 are as defined in Claim 13; and

P^1 is of the formula:

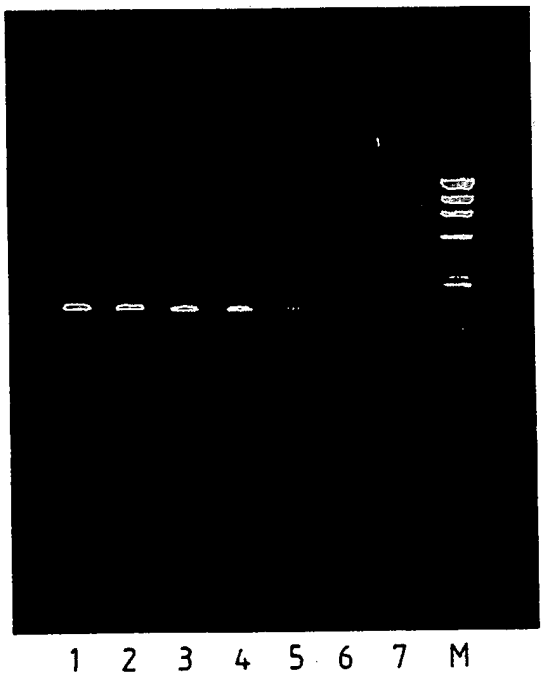


wherein;

R_6 is H or a protecting group; and

Z_3 is a protecting group.

FIG. 1



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01687

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 H 21/00 C 07 F 9/24

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 H 21/00 C 07 F 9/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,8910978 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 16 November 1989, see the whole document	17
Y		1-5

X	WO,A,8401779 (H. KÖSTER) 10 May 1984, see the whole document	17
A		1

Y	Nucleic Acids Research, volume 17, no. 15, 11 August 1989, IRL Press, M. Mag et al.: "Synthesis and selective cleavage of oligodeoxyribonucleotides containing non-chiral internucleotide phosphoramidate linkages", pages 5973-5988, see pages 5980-5982	1-5
	--- -/-	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08-01-1992	30 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

III. DOCUMENTS CONSIDERED T BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	EP,A,0067597 (BIOLOGICALS INC.) 22 December 1982, see the whole document	1-3,5
A	EP,A,0313219 (M.L. TECHNOLOGY VENTURES) 26 April 1989, see the whole document	1,13,17 ,18
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